

CRYOPRESERVATION OF SPERMATOPHORE OF THE FRESH WATER SHRIMP, *MACROBRACHIUM ROSENBERGII*

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ABSTRACT

Spermatophores were removed from the sternum of a female *Macrobrachium rosenbergii* immediately after mating, and equilibrated in either fresh water or physiological saline, both containing 10% glycerol. After 10 to 60 minutes equilibration, these spermatophores were pre-frozen in liquid nitrogen (LN₂) vapor and submerged in LN₂. The spermatophores were then thawed by directly putting them into warm water, and attached to the sternum of other females using a glue. Successful fertilization was observed in cases where the spermatophores were equilibrated in fresh water for 15–30 minutes. At 10 minutes equilibration, most of the spawned eggs were not fertilized. Furthermore, fertilized eggs were not obtained without pre-freezing, or where spermatophores were equilibrated in physiological saline.

INTRODUCTION

Although cryopreservation of gamete or embryo has been attempted and actually applied in mammals (Leverage *et al.*, 1972; Whittingham *et al.*, 1972), teleosts (Horton and Ott, 1976), and invertebrates (Dunn and McLachlin, 1973; Hughes, 1973; Asahina and Takahashi, 1978; Zell *et al.*, 1979), no report has been made on decapod crustacea. Attempts to preserve the spermatophore of the fresh water shrimp *Macrobrachium rosenbergii* have been made by Sandifer and Lynn (1980) and Chow (1982), but fertility only lasted for 24 h in the former while in the latter, the preservation period was extended to 9 days.

This paper presents results on the first attempt at cryopreservation of spermatophore of the fresh water shrimp *Macrobrachium rosenbergii* in liquid nitrogen (–196°C) and observations on successful fertilization using preserved spermatophore.

MATERIALS AND METHODS

Collection of spermatophore

Adult males and females of *M. rosenbergii* were maintained individually in 40-liter fresh water aquaria at 25–29°C. The mature female is distinguished by the fully developed ovary observed through the translucent exoskeleton. She also undergoes a pre-spawning molt. Mating followed after transferring the female, which had recently experienced a pre-spawning molt, to an aquarium containing a male. The spermatophore was deposited on the female sternum, and was carefully stripped immediately after the mating.

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Treatment and cryopreservation of spermatophore

Fresh water, prepared by filtering tap water through activated carbon, or physiological saline were used as basic media for equilibration of the stripped spermatophores. These media contained 10% (V/V) glycerol as cryoprotectant. The composition of physiological saline was adopted from Nagamine *et al.* (1980) and shown in Table I. The swelling condition of spermatophores, exposed to three kinds of media (fresh water, fresh water containing 10% glycerol, and physiological salines containing 10% glycerol) was observed. After 0, 10, 15, 30, 60, 120, and 180 minutes of swelling the spermatophores were carefully wiped with cloth or cotton and their weights were determined for all media used.

For cryopreservation, swollen spermatophores exposed to fresh water for 10, 15, and 30 minutes or to physiological saline for 30 and 60 minutes at room temperature (25–30°C) were removed from these media and transferred to 1 cm diameter dry glass test tubes. The test tubes were then pre-frozen in liquid nitrogen (LN₂) vapor for 0, 5, and 10 minutes before submerging them into LN₂. Spermatophores were thawed in tap water at 30°C. Artificial insemination techniques have been previously described (Chow, 1982).

RESULTS

Morphological observations of spermatophore and spermatozoa during equilibration in various media and after preservation

The extent of swelling of the spermatophores in various media was determined by their weight increase at 0, 10, 15, 30, 60, 120, and 180 minutes (see Fig. 1). As expected, considerable swelling was observed in fresh water, followed by fresh water containing 10% glycerol, then physiological saline containing 10% glycerol. Maximum swelling of spermatophore was observed after 2 h in all media, where weight increases were about 10× in fresh water, 6× in fresh water containing 10% glycerol, and 2.5× in physiological saline containing 10% glycerol.

Remarkable visible changes, namely, the softening of the protective matrix and the slight hardening of the adhesive matrix, were observed in spermatophores equilibrated in fresh water but not in physiological saline.

Light microscopic observation showed no visible deformities of the spermatozoa during equilibrations in these media and even after freezing and thawing.

Fertility of cryopreserved spermatozoa

Among 15 trials of inseminations using frozen-thawed spermatophores, fertilized eggs were observed in 8 trials (Table II). In two cases where the spermatophores

TABLE I

Macrobrachium rosenbergii physiological saline

NaCl	11.00 g
CaCl ₂ · 2H ₂ O	1.91
KCl	0.52
MgSO ₄ · 7H ₂ O	2.47
NaHCO ₃	0.17

The salts were dissolved in distilled water and brought up to 1 liter. The pH was adjusted to 7.6 with 1 N NaOH. (From Nagamine *et al.*, 1980).

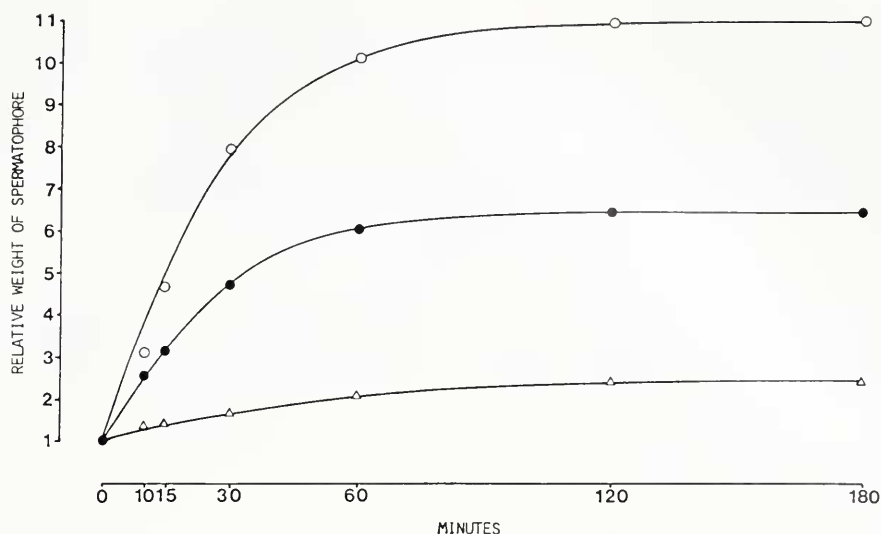


FIGURE 1. Fluctuation of spermatophore weight with the time elapsed after exposure to three kinds of media; fresh water (O), fresh water containing 10% glycerol (●), and physiological saline containing 10% glycerol (Δ).

were equilibrated for 10 minutes in fresh water containing 10% glycerol and pre-frozen for 5 minutes, most of the spawned eggs were not fertilized, and a few fertilized eggs had fallen off within a week together with the unfertilized eggs before growing to eyed-stage. In 7 combinations of 15 and 30 minutes equilibration in

TABLE II

Results of artificial insemination using shrimp spermatophore preserved in LN₂

Basic medium	Equilibration time (min)	Pre-freezing time in vapor LN ₂ (min)	Preservation period in LN ₂ (day)	Fertilization	Hatching
Fresh water	10	5	9	fertilized*	not hatched
	10	5	31	fertilized*	not hatched
	15	0	0.5	not fertilized	—
	15	0	26	not fertilized	—
	15	5	0.2	fertilized	hatched
	15	5	30	fertilized	hatched
	15	10	0.08	fertilized	hatched
	15	10	8	not fertilized	—
	30	5	0.13	fertilized	hatched
	30	10	0.17	fertilized	hatched
	30	10	20	fertilized	hatched
Physiological saline	30	10	0.04	not fertilized	—
	30	10	2	not fertilized	—
	60	10	1	not fertilized	—
	60	10	1	not fertilized	—

* A small number of fertilized and cleaved eggs were observed, and they had fallen off within a week.

fresh water with 5 and 10 minutes pre-freezing, 6 cases provided successful fertilization and hatching, while 2 cases of 15 minutes equilibration in this same medium with no pre-freezing were not fertilized. Furthermore, no fertilized eggs were observed in cases where the spermatophores were equilibrated in physiological saline for 30 and 60 minutes.

Under rearing conditions at 28°C, more than 70% of hatched larvae developed normally and metamorphosed into post larvae after a month.

DISCUSSION

Components of the basic media used in this study are simple when compared with the diluent for cryogenic preservation of spermatozoa in other animals studied. In contrast to their spermatozoa which are suspended in seminal plasma, the shrimp sperm are enveloped by the gel matrix (Berry, 1970; Bauer, 1976; Sandifer and Lynn, 1980; Chow *et al.*, 1982). The permeability of the gel matrix to fluids was demonstrated by the swelling of the spermatophore in the three media used. Successful fertilization occurred in cases where spermatophore was equilibrated in fresh water containing 10% glycerol, but not in physiological saline containing the cryoprotectant. This undoubtedly indicates that glycerol has a protective effect against freezing damage and that fresh water as basic medium was more effective in the transport of glycerol into the gel matrix. In physiological saline, the movement of glycerol into the gel matrix was not possible because the difference in osmotic pressure was minimal. This was shown by the slight swelling observed in physiological saline (Fig. 1).

While optimum cooling velocities are different in various tissues, rapid cooling above optimum rate generally causes intracellular freezing or recrystallization during thawing and results in death (Mazur, 1970). Although the cooling and thawing velocities were not extensively examined in this study, the results show that, in the two trials where pre-freezing was not done, fertilization did not occur; light microscopy did not detect any deformities of the spermatozoa. This indicates that rapid cooling may affect the spermatozoa.

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